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Authors: Jianguang Wang, Taesup Lee, Zhe Zhang, and Ching Tung

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A bioluminogenic probe for monitoring tyrosinase activity

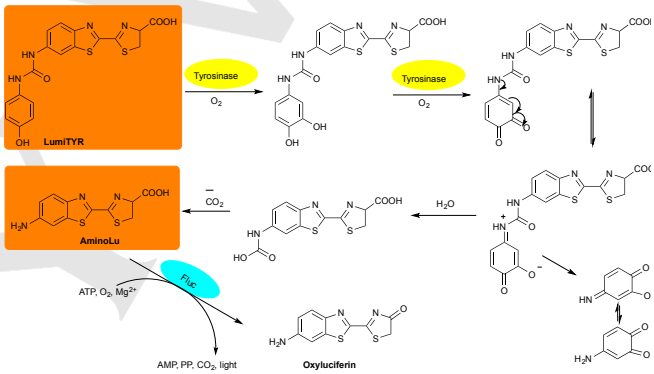
Jianguang Wang,[a][b] ‡ Tae Sup Lee,[a][c] ‡ Zhe Zhang,[a] and Ching-Hsuan Tung*[a]

Abstract: A novel bioluminogenic probe, based on luciferin, was designed and synthesized to monitor tyrosinase activity. Its applications were demonstrated in assessing tyrosinase activity in a buffered aqueous solution, and in measuring endogenous tyrosinase activity in melanoma cells.

Tyrosinase (TYR), a copper-containing monooxygenase enzyme, is widespread in fungi, plants, and animals. TYR has two oxidation enzyme activities: its monophenolase property catalyzes the hydroxylation of monophenol to *ortho*-diphenol, and its diphenolase property catalyzes the oxidation of diphenol to *ortho*-quinone in the presence of oxygen.[1] TYR plays a key role in the biosynthesis of melanin – the pigment that generates skin color, protects DNA in skin cells from ultraviolet, and scavenges reactive oxygen species.[2] TYR has been reported to be over-expressed in melanoma cells and a lack of TYR causes type I oculocutaneous albinism. In addition, excessive TYR can contribute to dopamine toxicity and neurodegeneration associated with Parkinson's disease.[3] TYR has been thus identified as a major target for medical and industrial applications, including cosmetic and agricultural industries.

The development of a sensitive and selective probe to monitor TYR activity has been an active research field over the past decade. A few TYR probes have been designed to monitor enzymatic activity by measuring fluorescent or electrochemical changes.[4] However, those methods are more suitable for biochemical analysis because they require external excitation or electricity. On the other hand, bioluminescence technique has been widely employed in biological assays, both *in vitro* and *in vivo,* due to its high specificity, wide dynamic range, and relative inexpensive instrument.[5] Many bioluminescent probes based on the luciferin-firefly luciferase system have exhibited excellent detection properties.[6] The non-invasive optical imaging of living cells or organs can be realized by detecting bioluminescent signals generated deep under skin tissue through a mechanism involving the reporter enzyme, firefly luciferase (FLuc), the substrate, luciferin, Mg^{2+} and ATP. Herein, we report the first bioluminogenic probe to monitor TYR activity.

To detect tyrosinase activity, a probe was designed based on the oxidative property of tyrosinase.[7] Aminoluciferin (AminoLu), which is slightly different from the natural luciferin structure, is known to undergo an oxidation reaction with FLuc; however, studies have also shown that FLuc is unable to recognize the amine derivatized AminoLu.[8] We, thus, envisioned that a TYR/FLuc dual detection could be achieved by employing a urea linkage to conjugate the monophenol motif to AminoLu. The proposed reaction mechanism is illustrated in Scheme 1. The oxidation of the monophenol moiety to *ortho*-quinone by TYR would trigger a spontaneous self-immolative elimination, freeing the caged AminoLu, which then could be oxidized by FLuc to form oxyluciferin and generate bioluminescence.



Scheme 1. The structure of LumiTYR and proposed reaction mechanism for the detection of tyrosinase activity.

The bioluminescent responsive probe, LumiTYR, was synthesized by adapting several reported protocols with modifications (detailed syntheses and characterizations were described in the Supplementary Information),[6d,9] The absorption, fluorescent and bioluminescent spectra of the prepared LumiTYR in PBS and in the presence of TYR were shown in Fig. 1 and Fig. S1. It is expected that in the presence of TYR, LumiTYR would be converted to AminoLu which could be further activated by FLuc. As shown in Fig. S1A & B, LumiTYR showed two characteristic maxima absorption peaks at 337 nm and 250 nm. Upon incubation with TYR for 12 h, the absorption peak at 337 nm broadened. It is known that the urea group could increase the solubility of the hydrocarbons through the hydrogen bond with water. It was thus postulated that the broadening of the peak could be due to the decrease in its solubility.[10] After incubation with TYR, the FLuc-induced bioluminescence (Fig. 1A) and fluorescence (Fig. S1C) were significantly enhanced. It is shown that the maximal fluorescence peak is around 517 nm and the maxima bioluminescence peak is around 600 nm. As displayed by the insert in Fig. S1C, LumiTYR alone did not exhibit any fluorescence; however, after a 12 h incubation with TYR, the mixture exhibited green fluorescence. Similarly, LumiTYR alone was unable to generate any

[a] Dr. J. Wang, Dr. T.S. Lee, Dr. Z. Zhang, Prof. C.-H. Tung
Molecular Imaging Innovations Institute, Department of Radiology,
Weill Cornell Medicine,
413 East 69th Street, New York, NY 10065, USA.
E-mail: cht2018@med.cornell.edu
[b] Current address: J. Wang
School of Chemical and Environmental Engineering,
Anyang Institute of Technology,
West of HuangHe Road, Anyang, 455000, PR China.
[c] Current address: T.S. Lee
Division of RI-Convergence Research,
Korea Institute of Radiological and Medical Sciences
75 Nowon-ro, Nowon-gu, Seoul, 01812, Republic of Korea
‡ These authors contributed equally to this work
Supporting information for this article is available: Experimental details, additional characterization studies, and additional information about experiments performed in cells.

bioluminescence unless it coexisted with both TYR and FLuc (Fig. 1A).

To validate the formation of the *ortho*-quinone intermediate by TYR triggered oxidation, an established MBTH (3-methyl-2-benzothiazolinone hydrazone) quinone-trapping assay was used (Fig. 1B, Supplementary information).[11] The pink color from the mixture of MBTH, TYR and LumiTYR is in agreement

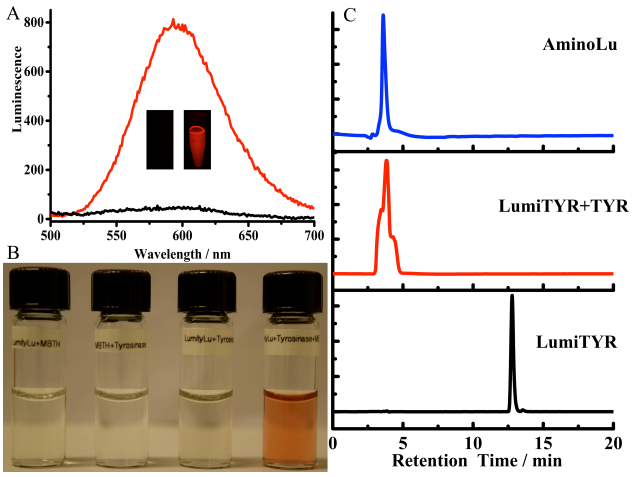


Fig. 1. (A) Bioluminescence response of LumiTYR towards TYR. The insert is a snapshot of bioluminescence. Black line and left image: 30 μM LumiTYR, Red line and right image: 30 μM LumiTYR incubated with TYR for 12h. Fresh prepared FLuc solution (0.43 μg/mL) was added prior the measurement. (B) MBTH color test based on TYR's oxidizing activity towards LumiTYR (from left to right: LumiTYR and MBTH; MBTH and TYR; LumiTYR and TYR; LumiTYR, TYR and MBTH), The mixture was incubated at 37°C for 3h. (C) HPLC retention time that generated by using an analytical HPLC column. The retention time of AminoLu, LumiTYR incubated with TYR for 12h, and LumiTYR is 3.80 min, 3.83 min, and 12.78 min, respectively.

with the literature, supporting the formation of conversion of the phenyl group to *ortho*-quinone intermediate.[4f] The LC-MS analysis of the reaction mixture also showed the correct molecular mass of the *ortho*-quinone intermediate (Fig. S2). Subsequently, the unstable *ortho*-quinone intermediate underwent a rapid self-immolative elimination to free AminoLu, which could be utilized by FLuc to generate bioluminescence in the presence of ATP and Mg^{2+}. To further confirm the proposed mechanism, AminoLu was synthesized as the standard. As shown in Fig. 1C, LumiTYR has a retention time of 12.7 min, which disappeared after its incubation with TYR; a new peak, corresponding to the formation of AminoLu, was observed at 3.8 min. The LC-MS analysis of the mixture also confirmed the formation of AminoLu (Fig. S2).

To examine the dose dependent reactivity, LumiTYR was incubated with different concentrations of TYR (0 - 114U) for 24 h at 37°C. Fig. 2A shows the fluorescence change of LumiTYR on titration of TYR. Without TYR, the fluorescence is at a background level. An increased concentration of TYR led to a higher fluorescence. Specifically, when LumiTYR was incubated with 114U/mL of TYR, the fluorescence was enhanced 27-fold. Under an optimized condition, a near linear correlation of fluorescence vs TYR activity was observed with 2 to 10 Units of TYR and LumiTYR (12 μM) in sodium phosphate buffer (Fig. S3). To generate bioluminescence, ATP, Mg^{2+} and FLuc was added to the mixture after a 24 h pre-incubation with TYR. The control sample, without the presence of TYR, did not produce a detectable bioluminescence even after incubation with FLuc for 24 h. On the other hand, samples of LumiTYR, which were incubated with different concentrations of TYR in advance and followed by the addition of FLuc, showed enhanced bioluminescence with increasing concentrations of TYR. The assay is found to be very sensitive. The bioluminescence could be detected even with only 0.057U of TYR. The bioluminescence was enhanced 25-fold when 114U/mL of TYR was used (Fig. 2B).

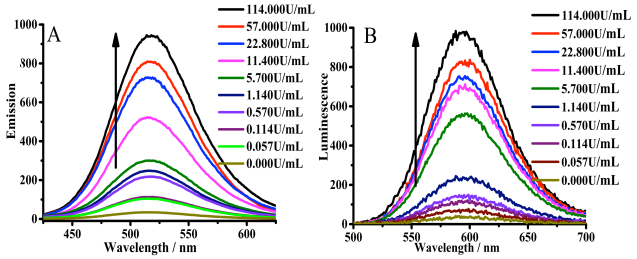


Fig.2. Fluorescent (A) and bioluminescent (B) spectra of LumiTYR (12 μM in sodium phosphate, pH=6.8) upon incubation with different concentration of tyrosinase for 24 h at 37°C. For bioluminescence measurement, fresh prepared FLuc solution (0.43 μg/mL) was added prior to the measurement.

The time dependent changes of the fluorescent and luminescent spectra of LumiTYR were also tested in the absence of TYR. As shown in Fig. 3, no fluorescence or luminescence was detected at 0 h, immediately after mixing the LumiTYR and TYR. Additionally, no spectrum was detected for the sample of LumiTYR in the absence of TYR after 9 h incubation, indicating that LumiTYR remained intact during incubation. When LumiTYR was incubated with 22.8 U/mL TYR for 1 to 9 h, the fluorescence and luminescence spectra were enhanced gradually. The luminescence signal intensity of the LumiTYR sample incubated with 22.8 U/mL TYR has exceeded the detection limit. The turn-on fluorescent and especially the turn-on luminescent properties of LumiTYR towards TYR suggested that LumiTYR is an excellent probe for TYR activity detection.

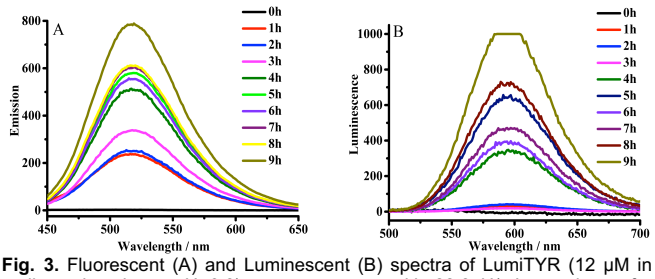


Fig. 3. Fluorescent (A) and Luminescent (B) spectra of LumiTYR (12 μM in sodium phosphate, pH=6.8) upon treatment with 22.8 U/mL tyrosinase for different time periods. For bioluminescence measurement, fresh prepared FLuc solution (0.43 μg/mL) was added prior to the measurement.

Since TYR is a copper-containing enzyme,[12] the interference of common metal ions other than copper, including Zn^{2+}, Fe^{3+}, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, was examined. The fluorescence and luminescence intensity of LumiTYR in the presence of different metal ions showed that the other metal ions could not interfere with the detecting property (Fig. S4). We then tested the fluorescent and luminescent signal of LumiTYR

at different pH environments. The reported optimal pH of TYR is between 6 - 7,[4] and the functional pH of FLuc is 7.44 - 8.14 with maximal activity at pH of 7.8.[13] The pH effect of the LumiTYR property in a 0.1M sodium phosphate buffer at pH values 5.8 and 8.0 were shown in Fig. S5. The excellent probing ability of LumiTYR was observed at pH 6.8, which is in agreement with the previous report.[6] This finding supports the potential usage of LumiTYR in a physiological condition.

To investigate the capability of LumiTYR for screening a TYR inhibitor, TYR was pre-incubated with a commonly used inhibitor[14], benzaldehyde, for 15 min at room temperature before its incubation with LumiTYR. As demonstrated in Fig. 4, the catalysing activity of TYR was significantly inhibited with 5 μM of benzaldehyde. When the sample of LumiTYR and TYR was pre-incubated with 10 μM benzaldehyde, an almost complete inhibition effect was observed. These results further verified that LumiTYR is a potential lumionogenic probe for TYR detection.

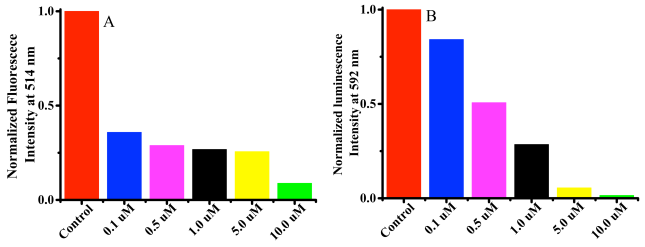


Fig. 4. Inhibition efficiency response of LumiTYR (12 μM in sodium phosphate, pH=6.8) upon treatment with 57U/mL tyrosinase and different concentrations (control, 0 μM) of inhibitor(benzaldehyde) for 12h.

To evaluate the applicability of detecting endogenous TYR activity in a living cell, LumiTYR was validated with B16-F10 melanoma cells that are known to overexpress TYR. Two commercially available MDA-MB-231 breast cancer cells (TYR-/FLuc-) and MDA-MB-231-Luc (TYR-/FLuc+) cells, which have no TYR activity, were used as the negative controls. The parent B16-F10 cells (TYR+/FLuc-) were first transduced with luciferase/RFP genes, and a stable clone was selected for the study. As shown in Fig. S6, the parental B16-F10 (TYR+/FLuc-) cells displayed no red fluorescence signal nor bioluminescence, while the transfected B16-F10-RF (TYR+/FLuc+) cells expressed red fluorescent protein and firefly luciferase which were verified by fluorescence imaging and a bioluminescence assay using D-luciferin as the substrate. An excellent correlation (R^2 = 0.993) between the bioluminescent signal intensity and the cell number was observed. LumiTYR was then tested with MDA-MB-231 (TYR-/FLuc-) MDA-MB-231-Luc (TYR-/FLuc+), B16-F10 (TYR+/FLuc-) and B16-F10-RF (TYR+/FLuc+). As demonstrated in the tyrosinase specific bioluminescence assay using LumiTYR, MDA-MB-231 (TYR-/FLuc-) and B16-F10 (TYR+/FLuc-) cells only showed a background level bioluminescent signal. MDA-MB-231-Luc bearing only FLuc showed a low level bioluminescent signal, but B16-F10-RF cells bearing both TYR and FLuc activity showed a greater bioluminescent signal, as compared to other cells using a luminometer (Fig. 5A). In bioluminescence imaging using LumiTYR, B16-F10-RF cells were successfully visualized by the tyrosinase triggered and firefly luciferase mediated bioluminescence process (Fig. 5B). The endogenous TYR alone in B16-F10 cells could convert LumiTYR to AminoLu, but was unable to generate bioluminescence. These results indicated that LumiTYR could monitor and image TYR activity in mammalian cells in the presence of FLuc. This tyrosinase specific bioluminescence assay using FLuc expressing B16-F10 cells and LumiTYR could be applied to screen various tyrosinase inhibitors.

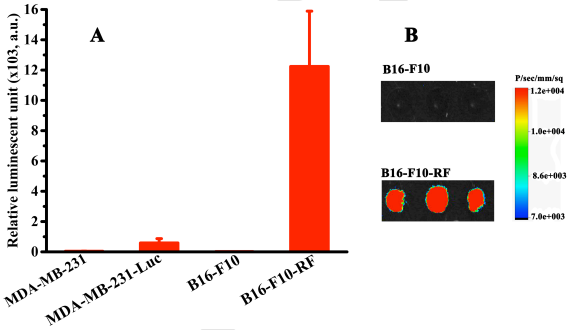


Fig. 5. Tyrosinase specific bioluminescence of various cancer cells using LumiTYR. (A) Bioluminescent signal intensity of MDA-MB-231 (TYR-/Fluc-), MDA-MB-231-Luc (TYR-/FLuc+), B16-F10 (TYR+/FLuc-), and B16-F10-RF (TYR+/FLuc+) cells. (B) Bioluminescence image of B16-F10 (TYR+/FLuc-), and B16-F10-RF (TYR+/FLuc+) cells in triplicate.

In conclusion, a novel bioluminogenic probe LumiTYR towards TYR detection was developed for the first time. The detecting activity of LumiTYR was characterized in vitro with mushroom tyrosinase and mammalian tyrosinase based assays. The detection mechanism of TYR activity is through TYR mediated oxidative reactions followed by FLuc catalyzed bioluminescence generation. In addition, LumiTYR is also fluorogenic. After reacting with TYR, the enzyme activity could be conveniently quantitated by measuring the fluorescence increase at 517 nm. Similar to the other reported TYR fluorescence probes, LumiTYR would be useful in cell free assays. However, the fluorescence probes have limited applications in biological systems, such as cells and animals, due to background, penetration and diffusion issues. The unique bioluminogenic capability of LumiTYR becomes handy. Because of this dual fluorogenic/bioluminogenic property, LumiTYR can be applied to detect the activity of TYR in cell free assay, cells, and, potentially, in animals. It could provide an efficient screening methodology of a tyrosinase inhibitor in biomedical and industrial fields.

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Keywords: Tyrosinase • Bioluminescence • Fluorescence, Probe • Luciferin

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